Chapter V

Efficacy of combinational supplementation of antioxidants and antimetastatic components against cancer employing *in vivo* cancer animal model
Summary and conclusions

- Antioxidant in aqueous extracts of BC - BCAE and SR – SRAE inhibited oxidative stress mediated CCl₄ induced hepatotoxicity.

- BCAE and SRAE also inhibited 20-methylcholanthrene induced cervix cancer.

- Pectic polysaccharide fraction of SR - SRPP showed potent (88 %) inhibition of lung metastasis induced by B16F10 melanoma cells in Swiss albino mice.

- Supplementation antioxidants from SRAE however were not as effective in inhibiting metastasis as that of SRPP alone.
5.6. Discussion

Cancer is a complex and multi-step mediated disease as revealed in earlier chapters and poses challenge in the management and cure. Despite several chemotherapeutic drugs available, since it causes toxicity also to the normal cells, it has its own limitations. Unfortunately due to immortal nature of cancer cells and their destined commitments to retain in the body resulted in execution of several metabolic pathways of the host leading to uncontrolled all physiological functions in the body irrespective of the type of cancer and finally causing difficulty for the survival of the affected person. Alternatives are hence essential to manage cancer patients, particularly in the advanced stages to overcome their sufferings. In this direction although several antioxidants have been shown to work potentially, they had their limitations in offering protection to host cell. Accumulated countless literature and our own results indicate that dietary/spices antioxidants can be good preventive agents than curative in nature. Mechanism of understanding of cancer spread from various workers and our laboratory is throwing some of ray of hopes to design metastatic blockers so that the life and regular lifestyle of the patient can be maintained. Extensive and commendable work from Dr. Raz’s group on identification of citrus pectin as a potential blocker for metastasis as evidenced by the inhibition of prostate cancer upon oral feeding of citrus pectin. Since dietary sources are potentially rich in antioxidants and pectic polysaccharides, in thesis, we examined the role of potent phenolic antioxidants and galectin-3 inhibitory polysaccharides from black cumin and swallow root. In this chapter we evaluated the in vivo efficacy of antioxidants in CCl₄-induced hepatotoxicity and 20-methylcholanthrene induced cervix models, where in both the cases the effect is due to the generation of oxidative stress. In vivo efficacy of the potential antimetastatic component from swallow root has been examined using widely accepted – B16F10 melanoma cells induced lung metastasis in Swiss mice.
5.6.1. In vivo effect of BCAE and SRAE against oxidative stress – mediated CCl₄ induced hepatotoxicity

Carbon tetrachloride – CCl₄ is known to produce free radicals, which damages hepatocytes. Damage of the liver can be due to 1. increased production of ROS that are generated to eliminate affected liver cells; 2. depletion of antioxidant – GSH and antioxidant enzymes. This imbalance in the antioxidant and oxidative stress status result in oxidation of lipids hence lipid peroxidation of the affected tissue – hepatocytes causing liver injury. The damage to liver can be measured by the elevated levels of SGPT, SGOT and ALP enzymes. In our experimental study we did notice elevated levels of SGPT and SGOT with the exception of ALP indicating liver injury. The spice extracts treated groups had significantly lowered SGPT and SGOT levels compared to CCl₄ induced groups indicating their protective effect. The antioxidants present in the serum were also evaluated using a stable free radical 1, 1, diphenyl-2-picryl hydrazyl. Compared to the healthy rats, the antioxidant activity in the serum of CCl₄ induced and spice extract treated groups were higher with no significant difference between them. Thus CCl₄ may induce the system to synthesize more antioxidant molecules to combat free radicals generated by CCl₄. In liver, CCl₄ treatment depleted the antioxidant enzymes namely CAT, SOD and POX leading to liver damage evidenced by higher TBARS levels. The depletion may be due to inhibition of enzyme synthesis and also damaging the enzyme itself making them inactive. The spice extract treatment has significantly reduced the deleterious effects of free radicals generated by CCl₄ treatment. Almost similar levels of antioxidant enzymes were seen in spice extract treated groups. Further, TBARS levels were almost equal to that of healthy animals indicating the beneficial role of BCAE and SRAE against CCl₄ induced liver toxicity.

In this study, CCl₄ caused an obvious decrease of GSH and antioxidant enzymes activity in liver homogenates, but the enzyme activity was higher in animals pretreated with BCAE and SRAE which are known to contain abundant phenolic acids with multi-potent antioxidant potency (Chapter – 2). Data thus may suggest that BC and SR extracts can ameliorate GSH and antioxidant enzymes or enhance its
activity by participating in biosynthesis in animals with CCl₄-induced liver injury.

SOD is one of the potent antioxidant enzymes in cells and catalyzes the conversion of superoxide ions into oxygen and hydrogen peroxide. In our study, SOD activity was dramatically decreased by CCl₄, but the effect was rescued in a concentration dependent manner by both BCAE and SRAE. SOD is an important antioxidant enzyme of antioxidant defense system for stabilizing oxidative reactions. Decreased activity and content of these enzymes cause the descent of antioxidant ability in the body. Therefore, antioxidant rich BCAE/SRAE may prevent experimental liver injury by modulating or enhancing GSH and SOD activities. Results are substantiated by the hepatoprotectivity by black cumin seed and swallow root, where the protectivity has been attributed to several compounds including thymoquinone, a major (27.8%–57.0%), component in black cumin seed oil and a cocktail of compounds like 2-hydroxy 4 methoxybenzaldehyde, vanillin, decalpine etc., in swallow root. In the current study however we attribute the hepatoprotective ability to mainly phenolic acids which are contributing significantly to antioxidant activity in addition to the reported compounds in SRAE. In black cumin however, since Ayurveda practitioners and traditional users use the water decoction, phenolics may contribute significantly to hepatoprotectivity in vivo. Increased binding ability to serum albumin may enhance the biopotency of these compounds since the rate of clearance of these phenolics may be delayed due to its binding ability to serum albumin and this may enhance the bioavailability of these compounds (Chapter – 4) and may be responsible for the distribution of these compounds to various tissues since HSA acts as a potent carrier protein which is known to carry such essential small molecular components.

It is concluded that both BCAE and SRAE may decrease the liver function enzymes and increase the antioxidant defence system activity in the CCl₄-treated rats. They may be therefore used in CCl₄-induced hepatotoxicity rats to prevent lipid peroxidation, increase anti-oxidant defense system activity and also to prevent liver damage.

Discussion
5.6.2 In vivo effect of BACE and SRAE against MC – induced cervix cancer

In view of the medicinal value attributed to black cumin and swallow root, the present study was an attempt to evaluate the effect of black cumin and swallow root aqueous extract on chemical induced carcinogenesis of uterine cervix in mice. As there are no reports on the chemopreventive effect of these spice extracts on chemical (20-methylcholanthrene) induced carcinogenesis on any organ or tissue so far, our study is perhaps the first report to demonstrate the anticarcinogenic activity of these spice extracts. In many studies the carcinogen is withdrawn after initiation, but in the present investigation we studied the effect during chronic carcinogenic exposure and therefore is of more significance with respect to assessment of the protective effect of black cumin and swallow root extract.

As a defense system against oxidative stress caused by reactive oxygen species, cells possess many endogenous non enzymatic antioxidant molecules including reduced glutathione as well as antioxidant enzymes such as superoxide dismutase, catalase and peroxidase (Somani et al., 1999; Lee et al., 2003). Hence, a large number of studies have focused on the pathogenic significance of oxidative stress in liver injury as well as on therapeutic intervention with oxidative scavengers.

Glutathione, the major endogenous antioxidant present in all animal cells, participate in diverse biological processes, including detoxification of xenobiotics for their elimination from the system and protection from oxidative stress (Sciuto, 1999). An observation on the association of GSH during carcinogenesis and in cancer is often contradictory and conflicting. A decreased level of GSH was reported in cervical neoplasia and invasive carcinoma (Kumar et al, 1995; Basu et al., 1991). Low plasma GSH was also observed in malignancies of breast, lung, liver, prostate and in lymphoma (Beuyer & Gilbert, 1985). In contrast to the GSH levels in serum, Meister and Griffith (1979) reports increased GSH content in the liver following carcinogen administration, which was in proportion to the carcinogenic potency. Our results showed a reduced serum and liver GSH level after 45 days of MCA exposure. It is
possible that initially the host’s defense system provides cellular protection by increasing the level of GSH activity, which helps in neutralizing the action of 20-methylcholanthrene. However, chronic exposure to MCA may derange the defense mechanism by significantly depleting the GSH level. Interestingly, oral administration of spice extracts elevated the GSH level resulting in delayed action of the carcinogen. The present observations suggest that the spice extracts induced increase in the level of GSH activity helps to eliminate the carcinogen and adjourn the onset of cervical neoplasia and its progression.

Several antioxidant enzymes like CAT, SOD and POX can protect cell and cellular DNA from oxidative damage. Cellular damages from radical and non-radical reactive oxygen species including peroxides and superoxides are inactivated enzymatically by CAT, SOD and POX (Vang et al., 1997). We observed a significant decrease in POX and CAT in both serum and liver samples in MCA treated group compared to normal. The reduction in SOD was significant in serum whereas it was marginal in the liver. Decreased cellular activities of POX, CAT and SOD causes accumulation of reactive species in the body, which leads to oxidative damage and progression of carcinogenesis. Spice extracts treatment significantly increased the POX, CAT and SOD activity (except in liver treated with BC1 and BC2 for SOD) in addition to the inductive effect on the production of SOD enzyme in the liver. This increased antioxidant enzymes facilitate the removal of peroxides and superoxides produced in large amounts during carcinogen metabolism, thereby preventing the oxidative damage and cancer initiation.

Generation of reactive species following increased lipid peroxidation and consequent tissue injury and cellular damage increases the risk for cancer (Wiseman and Halliwell, 1996; Swierczynski et al., 1997). A number of chemical carcinogens are known to increase formation of lipid peroxides. We observed elevated levels of lipid peroxides during MCA induced carcinogenesis in the uterine cervix. Decrease in lipid peroxides by black cumin and swallow root extracts in this study implies their chemopreventive action which is substantiated by observing the potent DNA
protective and cytoprotective activity of these extracts as revealed in Chapter - 2 which enables the prevention or arrest of DNA and cellular damage by the extracts. Wide array of protective activity may envisage the anticancer effect against MCA induced carcinogenesis and this may be attributed to potent phenolic antioxidants present BC and SR extracts.

These results were further substantiated by the morphological observation of the vagina and histological observation of the uterine cervix which, showed severe dysplasia in the MCA treated group, while black cumin and swallow root treated group (SR2) were found to be normal. The results of this study suggest that the potential chemopreventive activity of the black cumin and swallow root aqueous extract is partly attributable to their antioxidant properties.

5.6.3. Assesement of Antimetastatic activity of SRPP using B16F10 mouse melanoma cells on Swiss albino mice

The present investigation reports the role of swallow root pectic polysaccharide in the inhibition of metastasis in Swiss albino mouse model using B16F10 melanoma cells. B16F10 is a highly metastatic melanoma cell line reported by other investigators and our own analysis, and has often been used to investigate the efficacy of chemical agents in inhibiting metastasis or to understand the mechanism involved in the process (Fidler, 1973). Although the literature contains several references to the use of C57BL6J mice for inducing pulmonary metastases (Han et al., 2006; Kakuta et al., 2002; Menon et al., 1995; Banerji et al., 1998), we followed the method of Martinez et al., (2005) using Swiss albino mice, which were available in our laboratory.

Galectin-3 has been implicated in metastasis (Nangia-Makker et al., 2000) and the dietary intake of pectins rich in galactose have been reported to inhibit carbohydrate-mediated tumor growth, angiogenesis and metastasis in vivo (Nangia-Makker et al., 2002). Previously, we have investigated galectin inhibitory activity and inhibition of cell invasion by black cumin and swallow root pectic polysaccharides (SRPP) in vitro (Chapter – 2, Sathisha et al., 2007). Results indicated good activity for SRPP and
hence used for further studies on antimetastatic activity in vivo. SRPP contained high galactose and hence we compared with galactose-mediated inhibition of metastasis in vivo. Further, the role of polyphenols as antimetastatic and anticancer agent is available in the literature (Menon et al., 1995) and hence we included swallow root aqueous extract (SRAE), which contained higher polyphenolic content (Naik et al., 2007) to see its possible synergistic effect with SRPP. Swallow root contained 2-hydroxy-4-methoxybenzaldehyde as the major compound (Nagarajan et al., 2001), which is an isomer of methoxy, hydroxy benzaldehyde. Vanillin is also a similar type of isomer whose antimetastatic activity has been reported in the literature (Lirdprapamongkol et al., 2005). Also our own studies indicated in chapter – 2 suggest that HMBA is present in major amounts. Hence, the antimetastatic activity of this compound was also investigated in addition to SRPP and SRPP+SRAE.

Results from the present investigation shows significant reduction in lung metastases and also reduction in local tumor growth at the site of injection in mice treated with SRPP and SRPP+SRAE. HMBA did not show any correlation against lung metastasis. SRPP showed higher activity than the combinational supplementation of SRPP and SRAE. SRPP showed significant reduction of superficial tumor nodules, implantation percentage, growth index, and galectin levels indicating its antimetastatic ability. Although, SRPP+SRAE showed significant antimetastatic activity, it was comparatively less than SRPP alone. This may be due to the interaction of phenolic compounds with small oligomers of digested pectic polysaccharides resulting in lesser binding to galectin receptors. The binding ability of polyphenols to proteins may also have an effect on the reduced antimetastatic activity of combinational supplementation (SRPP+SRAE). Results also indicated significant increase in the serum antioxidant enzymes SOD, CAT and antioxidant GSH in SRPP treated animals indicating their stimulatory effect on the serum defense mechanism. The increase in serum antioxidant enzymes could be due to the presence of free phenolic antioxidants in SRAE and in the bound form with the polysaccharide. Our earlier studies on IR analysis of SRPP and other biochemical studies (srikanta et al., 2007) indicated the presence of 0.12 µg GAE phenol/g of
SRPP with potent antioxidant potency. Further, the increased levels of galectin in the positive control animals and reduction in animals treated with antimetastatic components correlated well with the severity of metastasis. These results further strengthen the view of galectin-3 involved in metastatic process as reported earlier (Inufusa et al., 2001). Toxicity studies indicated no significant damage to the vital organ liver as evidenced by no increase in the liver function enzymes (SGPT and SGOT) and also there is normal weight gain in sample treated groups as compared to healthy mice fed with standard mice feed. The decreased SGPT level is an added beneficial effect of the swallow root components in addition to their antimetastatic activity. There was no inhibition of metastasis by HMBA except that it had low toxic effects on the mice at the dosage used in the experimental study. These results may suggest the use of SRPP in inhibiting metastasis. Since swallow roots are consumed by people in South India in the form of pickles and as health drinks, SRPP may find use as a nutraceutical in controlling metastasis as it had no toxic effects. Further, the effectiveness of SRPP needs to be evaluated in higher animal models and finally in clinical trials.
5.5. Results

5.5.1 Effect of antioxidants in aqueous extracts of black cumin and swallow root on oxidative stress induced hepatotoxicity by CCl₄ in albino rat model.
Carbon tetrachloride (CCl₄) induced liver damage, and its protection by swallow root and black cumin-aqueous extract were carried out on experimental Albino Wistar rats. The serum analysis for total protein content indicated no significant difference between healthy, CCl₄ induced and spices extract treated groups (Figure 5.1D).

Effect on Liver function enzymes
The liver function enzymes namely SGPT, SGOT and ALP were analyzed in serum. -- to ---- fold increase in the activity observed in CCl₄ induced – stressed rats when compared with those of healthy rats. Both marginal and significantly altered levels of SGOT (1.4 fold increase) and SGPT (4.1 fold increase) [Figure 5.1A & C] were observed in CCl₄ treated group respectively. BCAE and SRAE could protect up to ~39 % and 72 % respectively indicating that SRAE at equal phenol concentration yielded ~ 2 fold better activity than BCAE. ALP levels among all the animal groups remained unaffected.

Biochemical changes in the serum and liver
TBARS levels (Figure 5.1F) in CCl₄ induced rats indicated 2.1-fold increase compared to healthy rats. TBARS levels although not improved upon treatment with BCAE, 62 % protection was observed with SRAE. Free radical scavenging activity (Figure 5.1E) in serum indicated 2.7-fold deprivation in CCl₄ treated groups while improved with > 80 % upon treatment with our selected sources - BCAE and SRAE.
Figure 5.1. Serum analysis for liver function enzymes, protein, DPPH radical scavenging activity and TBARS levels. Values are expressed as mean ± SD (n = 6). The analysis utilized student’s t-test to test each treatment group mean against control (healthy) mean.* Significant (p< 0.05); ** very Significant (p< 0.01); *** Highly Significant (p< 0.001) and NS – Non Significant.
In the liver although there were no significant alterations in total protein in the tissue, 2 fold increase of TBARS levels observed was normalized with the extracts (Figure 5.2A & B). All the antioxidant enzymes namely SOD, CAT and POX were significantly depleted in CCl₄ induced rats compared to healthy controls (Figure 5.3A-C). The spice extracts treated groups showed significant recovery of these enzymes to varying extent. BCAE treated groups showed 58, 50 and 58 % recovery of CAT, SOD and POX enzymes, respectively. SRAE treated groups showed 59, 62 and 58 % recovery of CAT, SOD and POX enzymes, respectively.

**Figure 5.2.** Biochemical analysis of liver homogenate. Values are expressed as mean ± SD (n = 6). The analysis utilized student’s t-test to test each treatment group mean against control (healthy) mean * Significant (p< 0.05); ** very Significant (p< 0.01); *** Highly Significant (p< 0.001) and NS – Non Significant.
Figure 5.3. Biochemical analysis of liver homogenate. Values are expressed as mean ± SD (n = 6). The analysis utilized student’s t-test to test each treatment group mean against control (healthy) mean * Significant (p< 0.05); ** very Significant (p< 0.01); *** Highly Significant (p< 0.001) and NS – Non Significant.
5.5.2. Effect of Antioxidants in aqueous extracts of black cumin and swallow root on 20-methylcholanthrene induced cervical cancer in albino mice model.

Severe inflammation and swelling due to cancer induction was observed near the vaginal surface of MC treated animals (Figure 5.4B); while upon treatment with extracts normalization was observed, similar to that of the untreated control upon treatment with SRAE. BACE showed only a marginal effect. Histological results in figure 5.4A of uterine cervix showed moderate to severe dysplasia in MC administered group. Following treatment with 100 mg GAE phenol/Kg b.w, of BCAE/SRAE, mild dysplasia was observed in only 20 % of the population, while there was no evidence of dysplasia in the remaining 80 % of animals. Administration of extract at 200 mg GAE phenol/Kg b.w showed changes which were comparable to that of normal healthy mice suggesting the dose dependent anticancer effect by antioxidants of BC and SR.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Histopathological observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>Normal</td>
</tr>
<tr>
<td>Positive control</td>
<td>Dysplasia</td>
</tr>
<tr>
<td>SR1 treatment</td>
<td>Mild Dysplasia</td>
</tr>
<tr>
<td>SR2 treatment</td>
<td>Normal</td>
</tr>
<tr>
<td>BC1 treatment</td>
<td>Mild Dysplasia</td>
</tr>
<tr>
<td>BC2 treatment</td>
<td>Normal</td>
</tr>
</tbody>
</table>

(A) Healthy  
(B) BC Treated  
(C) MC Treated  
(D) SR Treated

Figure 5.4. (A) Histopathological observation of cervix of mice induced for cervical carcinogenesis. (B) Morphological changes of vaginal opening of experimental mice after 45th day of treatment with BC & SR aqueous extract (BC2 and SR2; 200 mg GAE phenol/Kg b.w.)
5.5.2.1. Antioxidant and Antioxidant enzyme status in the liver and serum

Antioxidant – GSH and Antioxidant enzymes like superoxide dismutase, catalase and peroxidase levels were measured in both liver homogenate and serum of healthy, MC-induced cancerous animals and also groups of animals treated with BCAE and SRAE in different concentrations – 100 and 200 mg GAE phenol/Kg b.w. Data presented in Table 5.1 reveal that there was no significant alteration in SOD, while 2.1 to 3.4 fold depletion was observed in catalase and peroxidase in cancerous animals when compared to those of healthy animals. However upon treatment with SRAE/BCAE ~ 50 – 80 % protection was observed in a dose dependent manner, both in the liver homogenate [Table 5.1] and Serum [Figure 5.5]. Approximately 2 fold reduction in GSH levels in the MC treated animal groups was also normalized upon treatment with the selected extracts – BCAE and SRAE.

Data thus may suggest that despite differences in phenolic content and phenolic acid composition, and although in vitro efficacy of SRAE may be 4 fold higher than BCAE as revealed in Chapter – 1, in vivo efficiency of antioxidant rich aqueous extracts of black cumin and swallow root are more or less the same. This could be due to the presence of a major amount of tannic acid in BCAE, while HMBA in SRAE where TA is a potent antioxidant - fold better than HMBA with 50 % inhibition of free radical scavenging activity [IC$_{50}$ of 1.15 µg/mL as opposed to that of HMBA, which has an IC$_{50}$ of 213 µg/mL. However other phenolic acids such as gentisic and gallic acid which are good antioxidants with an IC$_{50}$ of free radical scavenging activity of 3 and 1.1 µg/mL respectively, also contribute to SRAE activity.
Table 5.1. Effect of black cumin and swallow root aqueous extract on liver antioxidant enzymes in 20-methylcholanthrene induced carcinogenesis in the uterine cervix of Swiss albino mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>CAT</th>
<th>SOD</th>
<th>POX</th>
<th>GSH</th>
<th>TBARS</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Healthy</td>
<td>3.05 ± 0.21</td>
<td>42.18 ± 0.391</td>
<td>1.17 ± 0.24</td>
<td>22.73 ± 2.73</td>
<td>0.794 ± 0.031</td>
</tr>
<tr>
<td>II MC-Induced</td>
<td>1.39 ± 0.16***</td>
<td>39.84 ± 0.78NS</td>
<td>0.34 ± 0.03***</td>
<td>11.82 ± 0.61***</td>
<td>0.988 ± 0.099**</td>
</tr>
<tr>
<td>III SR1 treated</td>
<td>1.72 ± 0.12***</td>
<td>44.53 ± 3.52NS</td>
<td>0.8 ± 0.10***</td>
<td>13.18 ± 0.76***</td>
<td>0.914 ± 0.049*</td>
</tr>
<tr>
<td>IV SR2 treated</td>
<td>2.39 ± 0.16***</td>
<td>51.95 ± 0.78***</td>
<td>1.39 ± 0.19**</td>
<td>18.79 ± 1.36***</td>
<td>0.875 ± 0.036**</td>
</tr>
<tr>
<td>V BC1 treated</td>
<td>2.07 ± 0.48***</td>
<td>35.05 ± 0.38**</td>
<td>0.50 ± 0.04***</td>
<td>16.26 ± 0.16***</td>
<td>0.851 ± 0.025**</td>
</tr>
<tr>
<td>VI BC2 treated</td>
<td>2.39 ± 0.42***</td>
<td>38.08 ± 0.31*</td>
<td>0.77 ± 0.02***</td>
<td>18.44 ± 0.17***</td>
<td>0.843 ± 0.048**</td>
</tr>
</tbody>
</table>

Values are means ± SEM (n = 6). The analysis utilized student’s t-test to test each treatment group mean against control (healthy) mean * Significant (p< 0.05); ** very Significant (p< 0.01); *** Highly Significant (p< 0.01) and NS – Non Significant.
Figure 5.5. Effect of black cumin and swallow root aqueous extract on serum antioxidant enzymes in 20-methylcholanthrene induced carcinogenesis in the uterine cervix of Swiss albino mice. Values are means ± SEM (n = 6). The analysis utilized student’s t-test to test each treatment group mean against control (healthy) mean * Significant (p < 0.05); ** very Significant (p < 0.01); *** Highly Significant (p < 0.01) and NS – Non Significant.
5.5.3. Effect of antioxidants of swallow root-HMBA and galectin-3 inhibitory polysaccharides-SRPP on B16F10 mouse melanoma cells induced metastasis

5.5.3.1. Macroscopic study

Inoculation of B16F10 cells in the lateral tail vein resulted in the formation of metastatic nodules in the lungs (Figure 5.6A). In addition, there were local tumors at the site of injection on the tail (Figure 5.6B). Marked differences in the tumor growth were observed on the tail of SRPP and SRPP+SRAE treated mice compared to the metastasis induced mice which were injected with only metastatic B16F10 melanoma cells without any treatment. There was no observable difference in the tumor growth on the tail in HMBA treated mice. When compared to that of metastasis induced mice. Further, we measured the quantifiable metastatic nodules in the lungs that indicate the successful harboring of metastatic cells in the lung. A nodular structure with blackish coloration that could be distinguished on the lung surface and which were sufficiently separated from each other to be counted individually, and present in several numbers in metastatic mouse and totally absent in healthy lungs of healthy group of animals also suggested the extent of tumor harboring in the lung for survival, to seek more oxygen. The count made of these superficial metastatic nodules is depicted in Figure 5.7. The group (II) injected with only B16F10 cells which served as a metastasis induced group showed metastatic nodules between 176 and 438 randomly distributed over the lung surface with a mean of 328.17 ± 86.04. Group IV treated with SRPP at 200 mg/kg b.w. showed a mean of 40.67 ± 6.05, which represents a significant (p = 0.0004) reduction of 88% metastatic nodules compared to that of metastasis induced group. Group VI treated with a combination of SRPP and SRAE at a dosage of 200 mg/kg b.w. of SRPP and 40 mg/kg b.w. of SRAE showed a mean of 108.17 ± 7.44 indicating significant (p = 0.0011) reduction of 67% than that of positive control. However, Group VIII treated with HMBA at 10 mg/kg b.w. showed no significant (p = 0.8229) difference in the superficial metastatic nodules (315.17 ± 91.12) formation compared to that of metastasis induced group, group - II.
Figure 5.6. Macroscopic characteristics of the pulmonary metastatic nodules (A) and local tumor growth on the tail at the site of B16F10 cells injection (B).
Figure 5.7. Frequency of the pulmonary metastatic nodules of experimental animal groups. MI – metastasis induced; SRPP- swallow root pectic polysaccharide treated group; SRPP + SRAE - swallow root pectic polysaccharide and aqueous extract treated group; HMBA – 2-hydroxy-4-methoxybenzaldehyde treated group. Values are expressed as mean ± SD (n = 6). * p < 0.05, ** p < 0.01, *** p < 0.001 and NS Non-significant, represent significant changes relative to positive control.

5.3.2. Microscopic study

The localization of the metastatic colonies varied widely and was greater in number at the subpleural level Figure 5.8. In the intra-parenchymatose region, they occurred basically around the capillary vessels and veins of the bronchioles and bronchi and were of greater volume than their subpleural metastatic nodules. Morphologically, they were constituted by solid accumulations of neoplastic melanocytes, and in the largest nodules frequently showed small areas of necrosis localized in the central areas with frequent inflammatory infiltrates around the periphery. The melanin pigment was observed as a blackish-brown colored accumulation.
Figure 5.8. Histopathological photographs of healthy (H), and metastasis induced (MI), SRPP, SRPP + SRAE treated and HMBA treated lungs (Swiss Albino Mice)

Results
5.5.3.3. Percentage of implantation
The results (Figure 5.9A) indicated an implantation percentage of 24.54 ± 7.08 for metastasis induced group (group II). Group IV showed 87 % reduction in the number of implants in the lung parenchyma, while 79 % reduction was observed in-group VI. A mean implantation percentage of 3.08 ± 1.38 and 4.98 ± 1.18 were observed for group IV and VI, respectively. The mean implantation percentage of both group IV and VI were significantly (IV – p = 0.0011, VI – p = 0.0013) different compared to metastasis induced group. Group VIII showed a mean invasion level of 20 % with statistically insignificant (p = 0.4599) reduction of implants (15.2 %).

5.5.3.4. Growth index
Figure 5.9B shows the growth index of various groups of mice studied for antimetastatic activity. Group II (metastasis induced group) showed a growth index of 0.0025 ± 0.0007, and Groups IV and VI had a growth index of 0.0009 ± 0.0004, 0.001 ± 0.0003, respectively. A statistically significant reduction in the growth index was also evident for groups IV (p = 0.0062) and VI (p = 0.0043). In HMBA treated group (VIII), there was no significant (0.0611) reduction in growth index (0.0015 ± 0.0004) observed.

5.5.3.5. Invasion index
The metastasis induced group (II) showed a mean invasion index of 97.45 ± 8.44, while Group IV and VI showed significantly (p < 0.001) reduced invasive index of 14.65 ± 3.13 and 32.72 ± 2.75, respectively (Figure 5.9C). Group VIII also showed a significant difference in the invasion index compared to the positive control with a higher invasion index value of 136.06 ± 10.40 indicating increased invasion of B16F10 melanoma cells to the lungs.
Figure 5.9. Implantation percentage (A), growth index (B) invasion index (C) and melanin levels (D) of pulmonary metastasis induced experimental mice. MI – metastasis induced; SRPP - swallow root pectic polysaccharide treated group; SRPP + SRAE - swallow root pectic polysaccharide and aqueous extract treated group; HMBA – 2-hydroxy-4-methoxybenzaldehyde treated group. Values are expressed as mean ± SD (n = 6). * p < 0.05, ** p < 0.01, *** p < 0.001 and NS Non-significant, represent significant changes relative to MI.
5.5.3.6. Galectin-3 levels in lung tissue

Galectin-3 as indicated earlier is a metastatic marker. When levels were determined by galectin-3 specific monoclonal antibody 2.5 fold increase in galectin-3 was observed (Figure 5.10A) in metastasis induced animals when compared to that of healthy lungs. Data thus may substantiate the results of tumor/invasion index. HMBA which did not reduce tumor index showed no alteration in the galectin-3 levels. Results thus support the role of galectin-3 in metastasis.

![Figure 5.10](image)

**Figure 5.10.** Lung galectin-3 (A) and melanin (B) levels in pulmonary metastasis induced experimental mice. MI – metastasis induced; SRPP – swallow root pectic polysaccharide treated group; SRPP + SRAE - swallow root pectic polysaccharide and aqueous extract treated group; HMBA – 2-hydroxy-4-methoxybenzaldehyde treated group. Values are expressed as mean ± SD (n = 6). * p < 0.05, ** p < 0.01, *** p < 0.001 and NS Non-significant, represent significant changes relative to MI.

5.5.3.7. Melanin content in lungs

Melanin is a chromogenic molecule produced specifically by melanoma cells. In this case it is from B16F10 mouse melanoma cells which were harbored in the metastatic lung, at very high levels. Healthy animal notably did not show melanin levels. Melanin content hence indicates the concentration of melanoma cells established in the lung. Data substantiates the results observed in histopathology analysis (Figure
5.10B).

5.5.3.6. Serum analysis for protein, antioxidant and antioxidant enzymes

Serum analysis (Table 5.2) indicated a total protein of 12.11 mg/mL in positive control group (II). Group IV and VIII showed significantly higher total protein content of 21.48 ± 2.18 and 17.45 ± 1.64 mg/mL, respectively. The total protein content in group I and group VI was not significantly different from that of positive control group.

Antioxidant enzyme SOD was depleted in metastasis induced group (1.86 ± 0.334) compared to healthy animal group (3.32 ± 0.129). Significant recovery of enzyme activity was observed in group IV (60 %), group VI (37 %) mice, while no recovery of enzyme activity (non-significant) was observed in HMBA treated animals (VIII). There was no significant difference observed in CAT level in healthy, metastasis induced group and SRPP+SRAE treated animals. However, increased (32.78 ± 2.72) level of enzyme activity was observed in group IV animals, while significant reduction (7.78 ± 2.52) was observed in group VIII animals. Around 18 % reduction was observed in GSH antioxidant in the metastasis induced group, while no difference was observed in group VI and VIII animals. The GSH level in group IV was almost similar to that of healthy group.

Results of toxicity studies indicated a gradual increase in the body weight of mice in control group III, V and VII similar to healthy animals (Fig. 5.11A). In addition, higher amounts of total protein (non-significant) compared to healthy group (I) was also observed in all the control groups. Liver function enzyme SGPT in all the three groups (III, V and VII) were almost similar to healthy animals. The SGOT levels were significantly (p < 0.05) lowered in all the three control groups.
### Table 5.2. Analysis of serum protein, antioxidant and antioxidant enzymes.

<table>
<thead>
<tr>
<th></th>
<th>Healthy</th>
<th>Metastasis induced</th>
<th>SRPP</th>
<th>SRPP+ SRAE</th>
<th>HMBA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Protein</strong></td>
<td>13.96 ± 4.14 NS</td>
<td>12.11 ± 1.35</td>
<td>21.48 ± 2.18 ***</td>
<td>15.2 ± 3.49 NS</td>
<td>17.45 ± 1.64 ***</td>
</tr>
<tr>
<td><strong>SOD</strong> (U/mg protein)</td>
<td>3.32 ± 0.129***</td>
<td>1.86 ± 0.33</td>
<td>2.73 ± 0.38**</td>
<td>2.40 ± 0.23*</td>
<td>2.01 ± 0.40 NS</td>
</tr>
<tr>
<td><strong>CAT</strong> (U/mg protein)</td>
<td>16.35 ± 3.64 NS</td>
<td>13.72 ± 4.89</td>
<td>32.78 ± 2.72***</td>
<td>10.87 ± 3.46 NS</td>
<td>7.78 ± 2.52**</td>
</tr>
<tr>
<td><strong>GSH</strong> (mg/mg protein)</td>
<td>2.20 ± 0.16*</td>
<td>1.81 ± 0.23</td>
<td>2.23 ± 0.26*</td>
<td>1.73 ± 0.55 NS</td>
<td>1.60 ± 0.49 NS</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD (n = 6). * p < 0.05, ** p < 0.01, *** p < 0.001 and NS Non-significant, represent significant changes relative to metastasis induced group.

### Figure 5.11. Effect of SR components on serum protein (A), weight gain (B), and liver function enzymes (C) of experimental Ctrl mice. Values are expressed as mean ± SD (n = 6). * p < 0.05, ** p < 0.01, *** p < 0.001 and NS Non-significant, represent significant changes relative to Healthy control.
5.4. Materials and Methods

5.4.1. Chemicals

1,1-Diphenyl-2-picryl hydrazyl (DPPH), Folin-Ciocalteau reagent, 2-thiobarbituric acid (TBA), hydroxylamine hydrochloride, hydrogen peroxide, Nitro blue tetrazolium (NBT), 2-thiobarbituric acid (TBA), hydroxylamine hydrochloride, hydrogen peroxide, glutathione reductase, NADPH, reduced glutathione (GSH), 5, 5’- dithionitrobenzoic acid (DTNB), butylated hydroxyl toluene (BHT), 1,1,3,3 tetramethoxypropane (TMP), and Tris-HCl, 20-methylcholanthrene, gallic acid, Dulbecco’s modified eagle’s medium (DMEM), glutamine, sodium bicarbonate, penicillin, kanamycin, FBS (fetal bovine serum), MTT (3-[4,5-dimethyl-2-thiazolyl]-2, 5-diphenyl-2-H-tetrazolium bromide), skimmed milk powder, paranitrophenyl phosphate, were purchased from Sigma Chemical Co. (St. Louis, MO). Twenty six and half gauge insulin syringe and monoclonal anti human galectin-3 antibody were purchased from Becton Dickinson Co., USA. Alkaline phosphatase conjugated-rabbit anti mouse IgG secondary antibodies was procured from GENEI, Bangalore, India. Other chemicals such as carbon tetrachloride, copper sulphate, sodium potassium tartarate, sodium carbonate, hexane, hydrochloric acid, trichloro acetic acid (TCA), phosphate buffer, sucrose, ethylenediaminetetraacetic acid (EDTA) and solvents used were of the analytical grade purchased from local chemical company, Sisco Research Laboratories, Mumbai, India.

5.4.2. Collection of sources and preparation

Antioxidant – rich fraction from black cumin – BCAE, swallow root – SRAE, and potent antimetastatic pectic polysaccharide from swallow root - SRPP were selected for in vivo studies. The total phenolic content in BCAE and SRAE were determined colorimetrically using Folin-Ciocalteau method (Singleton and Rossi, 1965), since antioxidants found were phenolics in nature. 2-hydroxy-4-methoxybenzaldehyde - HMBA was isolated from swallow root as pure crystals as per the protocol (Nagarajan et al., 2001), purity and identification of the compound was confirmed by NMR.
technique as revealed in Chapter – 2. Total carbohydrate content in SRPP was measured by phenol sulphuric acid method, as revealed in Chapter – 3.

5.4.3. Study of the effect of BCAE and SRAE on Oxidative Stress/CCl₄ induced - Hepatotoxicity in Albino wistar Rats

5.4.3.1. Animals and treatments

Female albino rats of Wistar strain weighing 90-150 g, were selected for the study. Animal experiments were carried out upon the clearance from the CFTRI Ethics committee, at the animal house facility of CFTRI, which has been registered with CPCSEA (Reg. No. 49, 1999), Government of India, New Delhi, India. Animals were obtained from the animal house of Central Food Technological Research Institute, India and were acclimatized to the laboratory conditions in a light and temperature controlled room for 15 days before starting the experiments in the same animal house. Animals received standard diet and water throughout the study. Animals were divided into four main groups of six animals in each group.

**Group I:** Control, normal healthy rats - H; **Group II:** CCl₄ induced where animals were challenged with CCl₄ orally (2 mL/Kg b.w.) on 16th day of the experimental schedule – CCl₄; **Group III:** BCAE pretreatment for 15 days followed by CCl₄ challenge (similar concentration as mentioned in Group II. Rats administered with BCAE orally 200 mg GAE phenol/Kg b.w.) for 15 days and challenged with CCl₄ on 16th day – BC; **Group IV:** SRAE pretreatment (200 mg GAE phenol/Kg b.w.) for 15 days followed by CCl₄ treatment (2 mL/kg b.w.) - SR. Rats administered with SRAE orally for 15 days and challenged with CCl₄ on 16th day. At the end of the experimental schedule, the rats were fasted for 24 h and all the animals were sacrificed as per the procedures approved by the Institutional Ethics Committee of CFTRI, Mysore, India.
5.4.3.2. Assessment of Hepatotoxicity by CCl₄; protection by BCAE/SRAE.

5.4.3.2.1. Preparation of serum and liver homogenates

All the animals after the treatment, were anaesthetized with diethyl ether. Serum was collected from blood samples. Liver was removed and five percent liver homogenate was prepared using cold 0.15 M potassium chloride and centrifuged at 5000 g for 20 min at 4 °C. The supernatants obtained were collected and used for the estimation of thiobarbituric acid reactive substances (TBARS), peroxidase (POX), catalase (CAT) and total protein (Lowry et al., 1951). Five percent liver homogenate using 0.25 % sucrose in phosphate buffer (pH 7.4, 20 mM) was employed for the estimation of superoxide dismutase (SOD).

5.4.3.2.2. Assessment of hepatomarkers/antioxidant and antioxidant enzymes

To find out the antioxidant or hepatoprotective effect of the sample, the following serum marker enzymes for liver function such as serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), and alkaline phosphatases (ALP) were analyzed as per the standard protocols. In addition, the total protein content, TBARS and radical scavenging activity in the serum were also analyzed. Further, antioxidant enzymes like SOD, CAT and POX were analyzed in addition to total protein and TBARS content in liver homogenate also.

Estimation of Superoxide dismutase (SOD, EC 1.15.1.1)

The activity of SOD was assayed using nitroblue tetrazolium (NBT) as the substrate (Flohe and Otting, 1984). Briefly 0.1 mL of 5 % liver homogenate in 0.2 M sucrose in phosphate buffer (pH 7.4) or serum was taken in Beckman quartz cuvette of 1 cm path length. To this, a mixture containing 1 mL of sodium carbonate (50 mM), 0.4 mL of NBT (24 µM) and 0.2 mL of EDTA (0.1 mM) was added and the zero min reading was taken at 560 nm. The reaction was initiated by the addition of 0.4 mL of
1 mM hydroxylamine hydrochloride. The reaction mixture was then incubated at 25 °C for 5 min and the reduction of NBT was read at 560 nm. A parallel control without homogenate or serum was also run and was considered as 100 % autoxidation. The enzyme activity was expressed as unit/mg protein.

Estimation of Catalase (CAT, EC 1.11.1.6)
The activity of catalase was assayed according to the method described previously (Aebi, 1984). Briefly, 0.1 mL of liver homogenate or serum was added to 1.9 mL of phosphate buffer, pH 7.0 and absorbance was measured at 240 nm. To this 1 mL of hydrogen peroxide was added and the absorbance was measured after 1 min at 240 nm using phosphate buffer as blank solution. The activity of catalase was expressed as units/mg protein (1 unit is the amount of enzyme that utilizes 1 µ moles of hydrogen peroxide/min).

Glutathione peroxidase (POX, EC.1.11.1.9)
The activity of glutathione peroxidase was determined according to the method described (Flohe and Gunzler, 1984). The mixture containing 0.1 mL liver homogenate or serum, 0.1 mL of 10 mM glutathione reductase (0.24 U) and 0.1 mL of 10 mM GSH was preincubated for 10 min at 37 °C and, thereafter 0.1 mL of NADPH solution was added. The hydroperoxide independent consumption of NADPH was monitored for 3 min. Overall reaction was started by adding 0.1 mL of prewarmed hydroperoxide solution and the decrease in absorption at 340 nm was monitored for 3 min and the activity was expressed as n moles of NADPH oxidized/min/mg protein.

Measurement of Thiobarbituric acid reactive substances (TBARS)
Thiobarbituric acid reactive substances (as malondialdehyde) in serum and liver homogenate were analyzed according to the method of Buge and Aust (1978). Briefly, 0.25 mL of liver homogenate or serum was mixed with 2 mL of TCA- TBA-HCl reagent (15 % TCA, 0.375 % TBA in 0.25 N HCl) containing 0.05 % BHT and heated for 15 min in boiling water bath. The solution was cooled to room temperature. The
precipitate was removed by centrifugation at 1000 g for 10 min at RT and the absorbance of the supernatant was measured at 532 nm. The amount of malondialdehyde was quantified using 1,1,3,3 tetramethoxypropane as standard. Antioxidant status in the serum was measured in terms of DPPH radical scavenging effect in serum samples.

5.4.3.3. Statistical analysis

All Experimental values are mean ± standard deviation (n = 6). One way ANOVA was carried out to find out the significant difference between the control and treatment groups followed by Dennett’s test. A value of $p < 0.05$, 0.01 and 0.001 was considered significant (*), very significant (**), and highly significant (***) $p$ value > 0.05 was considered non significant (NS).
5.4.4. Assessment of carcinogenicity by 20-Methylcholanthrene, protection by BCAE/SRAE.

5.4.4.1. Animals/Animal treatments

Swiss albino virgin female mice, in the age group of 5-6 weeks, weighing 25-30 g were maintained in the Animal House, CFTRI (CPCSEA, Reg. No. 49, 1999). The mice were categorized into six groups, each containing six animals caged in groups of 3 per cage and kept in alternating periods of light and dark conditions of 12 h each. The animals were allowed to acclimatize to the animal house conditions for 1 week and were fed with standard rat pellet feed and water was provided ad libitum. Animal body weight was checked regularly and dosage of extracts were calculated based on the body weight of animals and administered orally at indicated concentration for different groups of animals as per the following experimental design. Group I (n = 6) - Healthy control group (H): All animals were administered with normal saline once daily for 45 days; Group II (n = 6) – MC-induced (MC) where animals received MC only into the uterine cervix through the vaginal opening, at a dose of 10 mg/kg b.w. for 45 days. Group III/Group IV (n = 6) – BCAE - treated groups - BC1/BC2 where animals were administered with BCAE at 100/200 mg gallic acid equivalent (GAE) phenol/kg b.w., for BC1 and BC2 respectively, once daily for 45 days along with 20-methylcholanthrene treatment; Group V/Group V1 (n = 6) – SRAE treated groups - SR1/SR2: where animals were administered with SRAE at 100/200 mg GAE phenol/kg b.w., for SR1 and SR2 respectively, once daily for 45 days along with 20-methylcholanthrene treatment. Tolerance of BCAE and SRAE by the mice was determined using three groups of six mice each. The first group formed the control and the second group was administered with 100 mg GAE phenol/Kg b.w. of BCAE and SRAE extract and the third group received 200 mg GAE phenol/Kg b.w. of BCAE and SRAE orally once a day for 45 days. The body weights of the animals were recorded at 3-day intervals. During each experiment, food and water consumption was checked daily. The mice were observed for behavioral responses and mortality. At the end of the study, the animals were sacrificed and the weight of the liver, lungs, heart, thymus, spleen and kidney were determined.
5.4.4.2. Assessment of chemopreventive action of BCAE and SRAE

At the end of 45th day, all the animals were sacrificed by cervical dislocation following the procedures approved by the Institutional Ethics Committee of CFTRI, Mysore, India. Sacrificing was done swiftly to ensure minimal and negligible stress to the animals. The serum and liver samples were collected and the protein concentration was determined according to the method of Lowry et al, (1951) and assayed for antioxidant enzymes. Further, the uterus was removed, sectioned and stained with hematoxylin and eosin to detect the occurrence of neoplasia.

The liver was weighed and homogenized in chilled Tris-HCl buffer [10 mM, pH 7.4] at a concentration of 5 % (w/v), centrifuged at 5000 g at 4 °C for 20 min using high speed cooling centrifuge (Remi C 24, India). The clear supernatant was used for the estimation of lipid peroxidation products (TBARS as malondialdehyde - MDA), reduced glutathione (GSH), peroxidase (POX), catalase (CAT) and superoxide dismutase (SOD).

5.4.4.2.1 Measurement of antioxidant and antioxidant enzyme

Glutathione (GSH) content of liver homogenate and serum was determined as described elsewhere (Das et al., 1997; Sedlak and Lindsay, 1968). One milliliter aliquot of homogenate was mixed with equal volume of 10 % TCA and protein precipitate was removed by centrifugation at 10,000 g for 20 min at RT. The supernatant was added to equal volume of 0.5 M Tris-HCl, pH 9 containing 20 mM DTNB to yield yellow chromophore of thionitrobenzoic acid (TNB), which was measured at 412 nm. GSH was used as a reference standard. Antioxidant enzymes namely, CAT, SOD, POX and lipid peroxidation products-TBARS were analyzed as per the methods described earlier (Section 5.4.3.2).
5.4.5. Assessment of Antimetastatic activity of SRPP using B16F10 mouse melanoma cells on Swiss albino mice

5.4.5.1. Growth and maintenance of B16F10

B16F10, a highly metastatic sub-line, of the murine B16 melanoma was procured from National Center for Cell Sciences, Pune, India. Cells were cultured with DMEM—high glucose (4.5 g/L) with 4 mM glutamine, buffered to pH 7.2–7.4 and supplemented with 1.5 g/L sodium bicarbonate, penicillin (100 Units/mL), kanamycin (0.1 mg/mL) and 10 % fetal calf serum at 37 °C, in a humidified chamber with 95 % air and 5 % CO₂. Cultured cell growth and survival was monitored by MTT assay [21]. Cells (2.8×10⁴ cells/mL) were cultured with the specified medium in a 96 well microplate and after 72 h, 25 μL of MTT solution (5 mg/mL) was added, incubated at 37°C for 4 h. Hundred microliters of lysis buffer was added and cells were continued to incubate at 37 °C overnight (about 16 h) to dissolve the dark blue crystals and absorption of formazan solution was measured at 570 nm in a microplate reader (Spectra Max-340, Molecular Devices, Germany).

5.4.5.2. Animals and Animal treatments

Animal experiments were carried out upon the clearance from the Central Food Technological Research Institute Ethics committee, at the animal house facility of CFTRI, Mysore 570 020, India, which has been registered with CPCSEA (Reg. No. 49, 1999), Government of India, New Delhi, India. Forty eight female Swiss albino mice, 10–12 weeks old weighing between 28–36 g at the beginning were used for the experiment. Food and water was administered ad libitum during the study.

Each animal was injected with 5 x 10⁵ cells/200 μL of phosphate buffer saline (PBS) in the lateral vein of the tail. Treatment with SRPP, SRPP+SRAE, HMBA and PBS as control solution was provided for twenty days before inoculation and for another 21 days following inoculation with B16F10 cells. Groups are as follows; **Group I:** Healthy - PBS treatment-**H**; **Group II:** Metastasis induced with B16F10 cells-**MI**;
**Group III**: SRPP Control - 200 mg/Kg b.w. of SRPP alone; **Group IV**: SRPP treated - 200 mg/Kg b.w, challenged with B16F10 cells—SRPP; **Group V**: SRPP+SRAE Control - 200 mg kg-1 b.w. SRPP + 40 mg kg-1 b.w. SRAE (Antioxidant rich) alone; **Group VI**: SRPP+SRAE – treated - 200 mg/Kg b.w. SRPP + 40 mg/Kg b.w. SRAE challenged with cells—SRPP+SRAE; **Group VII**: HMBA control, 10 mg/Kg b.w. HMBA alone; **Group VIII**: HMBA- treated - 10 mg/Kg b.w. HMBA treatment challenged with cells—HMBA. Control groups - Group III, V and VII were used to study the toxicity effect of compounds under investigation. At the end of the experiments animals were sacrificed by cervical dislocation; serum was collected and analyzed for biochemical parameters. Lungs were excised and the metastatic nodules were quantitatively evaluated by two observers using the following models: In addition tumors if appeared in tail, the site of injection or elsewhere in the body are noted since it indicates the extent of metastatic spread.

**Model 1**: macroscopic study by stereoscopic magnifying glass (Olympus), counting the metastatic nodules of the pleural surface of the five lobules; **Model 2**: quantitative analysis at microscopic level of five sections of each lobule. Images at described magnification were captured by inverted microscope attached to an image analyzer (Leica DMLS model, Germany), with which the study regions were interactively selected. Following Martinez et al. [2005], the initial parameters evaluated were: the area of each complete lobule was obtained by capturing the image at 21x magnifications. The area of the metastases within each lobule and their maximum and minimum diameters were obtained by capturing the image at 55x magnifications and the following parameters were calculated:

1. **Percentage of implantation** (area of metastasis per lobule/total area X 100)
2. **Growth index** (mean area of metastasis/total area) and
3. **Invasion index** (area of metastasis per lobule/mean area of metastasis).
5.4.5.3. Biochemical analysis in the serum and lung tissue
Serum of all test groups were analyzed for total protein (Lowry et al., 1951), antioxidant enzymes such as SOD (EC 1.15.1.1) CAT (EC 1.11.1.6) and antioxidant GSH employing protocols described earlier (Section 5.4.3.2). Enzyme activity for SOD and CAT are expressed as units U/mg protein. To evaluate the toxic effects of SRPP components, serum of control groups were analyzed for total protein and liver function enzymes like serum glutamate pyruvate transaminase (SGPT) and serum glutamate oxaloacetate transaminase (SGOT) as per the standard protocols.

5.4.5.4. Preparation of lung homogenates
Lung tissue was excised from the mice and five percent liver homogenate was prepared using cold 0.15 M potassium chloride and centrifuged at 5000 g for 20 min at 4 °C. The supernatants obtained were collected and used for the estimation of galectin-3 and melanin content.

5.4.5.5. Determination of galectin-3/and melanin in Lung homogenate
Galectin-3 has been reported earlier that it is a metastastic marker (Nangia-Makker et al., 2000; Sathisha et al., 2007). ELISA was performed for the detection of galectin-3 levels in the lung tissue homogenate employing the protocol standardized previously (Rajeshwari et al., 1998). Monoclonal anti human galectin-3 antibody was employed at dilutions 1: 1,000 as primary antibody. Alkaline phosphatase conjugated rabbit anti mouse IgG at 1:5,000 dilution followed by paranitrophenylphosphate were used as secondary antibody and substrate, respectively. The absorbance was measured at 405 nm in a microplate ELISA reader (Molecular Devices, Spectramax 340, Germany).

The melanin content of lung tissue homogenates were measured according to the method of Oka et al. (1996) with slight modification. The liver homogenate is solubilized in boiling 1 M NaOH for 10 min and the spectrophotometric analysis of melanin content was performed at an absorbance of 400 nm. The entire experiment was performed in triplicate, and results were confirmed by three independent
experiments.

5.4.5.6. Histopathology
The lung tissue of mice were removed carefully, the weights recorded, fixed in 10% formalin in PBS, and then embedded in paraffin. The paraffin embedded tissue blocks were sectioned using a microtome and the thickness were set between 6-8 microns, the tissue sections were fixed on to the microscopic slide and stained with hematoxylin and eosin as per the standard protocol (Thompson and Samuel, 1966).

5.4.5.7. Statistical analysis
All results are expressed as mean ± standard deviation (n = 6). The analysis utilized student’s t-test to test each treatment group mean against control mean. A value of $p < 0.05$, $p < 0.01$, $p < 0.001$ and was considered significant, very significant, and highly significant, respectively. Statistical evaluation of the data was done using SPSS version 10.0 for Windows.
5.3. Introduction

Reactive oxygen species generated by various sources are known to damage cells, initiate cancer including various other chronic diseases. Cancer however as highlighted earlier appear to be one of the most complex disease to devise a strategy for cure and hence is life threatening. As a result there is a constant raise in cancer incidence as per cancer statistics. Our earlier chapters enabled us to explore the anticancer potential in selected spices – Black cumin and Swallow root since they showed multi-potent antioxidant activities with effective inhibition of lipid peroxidation, cellular oxidation, DNA oxidation/damage, protein oxidation etc (Chapter 2). Results indicated that aqueous extracts – BCAE and SRAE possessed potent phenolic acids contributing to the multi-potent antioxidant activity. Despite several knowledge and application of antioxidants against cancer, due to their diverse effect, search is constantly on by various investigators for potent antioxidants particularly from dietary/spice sources. Innumerous amount of literature provides the cancer preventive role of flavonoids and hydrophobic antioxidant principles such as quercetin, curcumin, capsaicin etc. Our laboratory since continuously showed major contribution of antioxidant activity particularly in aqueous extracts from phenolic acids, and showed that these can attribute to health beneficial properties as highlighted in chapter – 2, in the current chapter to substantiate their further application, we examined the in vivo efficacy using accepted animal models. Role of spices / dietary antioxidants are still the choice of today since they are non-toxic and is partly supported with epidemiological data. In addition, we have found a novel pectic polysaccharide in black cumin and swallow root, but later being the best in inhibiting one of the most deleterious step in cancer – cancer spread / metastasis as dealt in chapter 3. Current chapter also envisages the determination of in vivo efficacy of the potent swallow root pectic polysaccharide (SRPP) in delaying or preventing metastasis. In order to study the in vivo efficacy of both antioxidants and anti-metastatic compounds that are essential to be anticancer, following models were selected –Effect of 1. antioxidants against lipid peroxidation caused hepatotoxicity by CCl4; 2. against oxidative stress induced by 20 - methyl cholantherene, a carcinogen that causes cervix cancer and 3. against B16F10 mouse melanoma cell induced lung
metastasis in Wistar rats and albino mice respectively. Macroscopic, microscopic, immuno/biochemical/enzyme assays and disease apoptotic markers and histopathological analysis were performed to understand the potential in vivo effect of antioxidants and antimetastatic compounds from selected sources in selected models.
Antioxidant activity

Swiss Albino Rats
Liver Injury

SRAE/BCAE

20-Methylcholanthrene

Cervical Cancer

SRAE/BCAE

Anticarcinogenic activity

Swiss Albino Mice

SRPP/SRAE

Antimetastatic activity

B16F10 Mouse melanoma cells

Lung Metastasis

SRAE – Swallow root aqueous extract; BCAE – Black cumin aqueous extract; SRPP – Swallow root pectic polysaccharide

5.2. Work concept
5.1. Hypothesis

Chapter 2 and 3 provides a multi-potent antioxidant components in aqueous extracts of black cumin (*Nigella sativa*)-BCAE, swallow root (*Decalepis hamiltonii*)-SRAE, and a potent galectin-3 blocker from swallow root (SRPP), which is 14 fold better than the reported only source of galectin-3 blocker-citrus pectin. Chapter 4 provides their probable in vitro effect as they showed effective interaction with human serum albumin and DNA which potentially depicts the protection ability and bioavailability. Effect of galectin-3 blockade on inhibition of invasion and triggering of apoptosis by SRPP also has been highlighted. Current chapter addresses where such biochemically potent, *in vitro* active antioxidants and antimetastatic polysaccharide can work effectively also *in vitro* in appropriate model systems.